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Identification of an NTPase motif in classical swine fever virus NS4B protein

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ABSTRACT

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine caused by CSF virus (CSFV), a positive-sense single-stranded RNA virus within the *Pestivirus* genus of the *Flaviviridae* family. Here, we have identified conserved sequence elements observed in nucleotide-binding motifs (NBM) that hydrolyze NTPs within the CSFV non-structural (NS) protein NS4B. Expressed NS4B protein hydrolyzes both ATP and GTP. Substitutions of critical residues within the identified NS4B NBM Walker A and B motifs significantly impair the ATPase and GTPase activities of expressed proteins. Similar mutations introduced into the genetic backbone of a full-length cDNA copy of CSFV strain Brescia rendered no infectious viruses or viruses with impaired replication capabilities, suggesting that this NTPase activity is critical for the CSFV cycle. Recovered mutant viruses retained a virulent phenotype, as parental strain Brescia, in infected swine. These results have important implications for developing novel antiviral strategies against CSFV infection.

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Introduction

CSF is a highly contagious and often fatal disease that affects swine throughout various regions of the world. The etiological agent, CSFV, is an enveloped virus that belongs to the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005). The CSFV genome is a positive-sense single-stranded RNA that encodes a single polyprotein precursor that is co- and post-translationally processed by cellular and virus-encoded proteases to produce four structural (C, E^{rns}, E1, and E2) and 8 non-structural (NS) proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Rumenapf et al., 1993; Lindenbach et al., 2007; Thiel et al., 1996). Replication of Pestivirus viral genome is suggested to occur in close association with cytoplasmic membranes through the synthesis of a negative-stranded full-length genome (Gong et al., 1996; Lindenbach et al., 2007). This function is mediated by the activity of NS proteins. NS5B is a viral RNA-dependant RNA polymerase (Steffens et al., 1999; Xiao et al., 2002, 2006); its activity *in vitro* is enhanced by the presence of NS3 protein (Wang et al., 2010). NS3 has multiple functions and is essential for virus replication (Xu et al., 1997). The serine proteinase activity of NS3 is responsible for cleavage of NS4A, 4B, 5A, and 5B and requires the 64 amino acid NS4A protein as a cofactor (Xu et al., 1997; Tautz et al., 1997; Moulin et al., 2007). Additionally, NS3 possesses both nucleoside triphosphatase (NTPase) and RNA helicase activities (Suzich et al., 1993; Tamura et al., 1993; Wen et al., 2007, 2009). The RNA helicase-active portion of NS3 contains a nucleotide-binding motif (NBM) characteristic of all helicases and numerous NTPases (Walker et al., 1982; Gorbalenya and Koonin, 1989). The role of NS2 and NS5A in CSFV replication is less understood. *In vitro* studies using CSFV RNA replicons showed that NS2 is not essential for replication although its presence increased the persistence of RNA replicons in transfected cells (Moser et al., 1999). The role of NS5A in replication may involve at least two different activities: the stimulation of virus replication via formation of multisubunit replication complexes and the inhibition of virus translation (Xiao et al., 2009; Sheng et al., 2010).

The role of NS4B in CSFV replication remains unclear. In Hepatitis C Virus (HCV) and other related flaviviruses, NS4B was found to be an integral hydrophobic membrane protein required for the assembly of "membranous webs" that are derived from endoplasmic reticulum (ER) or the Golgi apparatus and are important for RNA replication (Hugle et al., 2001; Egger et al., 2002; Kim et al., 2004; Miller et al., 2006; Weiskircher et al., 2009). Einav et al. (2004) have shown that HCV NS4B contains a NBM which is conserved among HCV genotypes. This structural motif binds and hydrolyzes ATP, GTP and GDP and also possesses adenylate kinase activity (Einav et al., 2004; Thompson et al., 2009). Amino acid substitutions in the NBM of HCV NS4B impaired NTP binding and hydrolysis and resulted in decreased NTPase and adenylate

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kinase activity (Einav et al., 2004; Thompson et al., 2009). Furthermore, the NBM was shown to be critical for the role of NS4B during *in vitro* HCV replication (Einav et al., 2004; Lindstrom et al., 2006) and in cellular transformation and tumor formation (Einav et al., 2008).

Conserved sequence elements in NBM-containing proteins that possess NTPase activity harbor characteristic Walker A and Walker B motifs. The Walker A motif consists of a G-rich phosphate-binding loop with a consensus sequence G/AXXXXGKS/T (where X could be any residue) that is involved in binding of β- and γ-phosphates of NTPs. The Walker B motif consists of an Asp residue preceded by a stretch of hydrophobic amino acids (h) hhhhD or hhhhDD/E, that chelates the Mg^{2+} of the Mg–NTP complex (Walker et al., 1982; Gorbalenya and Koonin, 1989; Mimura et al., 1991; la Cour et al., 1985; Pai et al., 1989). In this study, data is presented demonstrating that CSFV NS4B has NTPase activity. *E. coli* expressed His-tagged CSFV NS4B purified protein hydrolyzes both ATP and GTP. Conserved Walker A and B motifs, characteristic of NBMs in CSFV NS4B (residues 209–216 and 335–342, respectively), are shown to be highly conserved in NS4B proteins of the other pestiviruses, Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV). It is also demonstrated that the enzymatic activity of the protein is affected by specific residue substitutions within identified Walker A and B motifs. Additionally, we have observed that NS4B of pestiviruses lacks the universally conserved K residue in the GKS/T signature of A motifs, but a highly conserved K_{206} residue is located at the N-terminal end of the canonical motif that has an effect on the NTPase activity of the protein. Additional results suggest that specific substitutions within the canonical Walker A and/or Walker B motifs in NS4B are deleterious for CSFV, while a CSFV Walker A revertant virus or viruses harboring mutations at K206 were viable and retained a virulent phenotype in infected swine.

Results

Identification of a nucleotide-binding motif in NS4B

Conserved sequence elements in NBM-containing viral proteins include both Walker A and Walker B motifs (Walker et al., 1982; Gorbalenya and Koonin, 1989) (Fig. 1). Amino acid sequence analysis of NS4B proteins showed that pestiviruses possess sequences resembling canonical Walker A (G/AXXXXGKS/T) and Walker B (hhhhD or hhhhDD/E) motifs (Figs. 2 and 3A). These sequences are located towards the C-terminal portion of CSFV NS4B, encompassing amino acid residues 209–216 and 335–342 of the protein, for A and B motifs respectively. Furthermore it was observed that the putative Walker A motif in pestivirus NS4B lacks the universally conserved K residue in the GKS/T signature sequence, where K has been substituted to V or I (Figs. 2 and 3A). Instead, NS4B of these viruses has a conserved K residue (K206 in CSFV) at the N-terminal end of the Walker A motif, resembling the position of the K residue in deviant Walker A motifs found in ATPase catalytic centers of phage terminases (Mitchell and Rao, 2004) and some prokaryotic and yeast DNA-dependent ATPases or GTPases (Koonin, 1993a, 1993b) (Fig. 3B). The putative Walker B motif in CSFV NS4B, $LLGVDSEG_{335-342}$, concurs with the consensus hhhhD (Fig. 3A). The motif is also conserved among other pestiviruses (Fig. 3A). The sequence $DSEG_{339-342}$ within the B motif matches the consensus DXXG found in highly conserved GTP-binding protein families including elongation factors, ras p21, phosphoenolpyruvate carboxykinase and guanine nucleotide-binding proteins of adenylate cyclase (G proteins) (Dever et al., 1987) (data not shown).

CSFV NS4B has NTPase activity

His-tagged CSFV strain Brescia NS4B protein was expressed in *E. coli* (Fig. 4A), and purified to near homogeneity using cobalt immobilized affinity chromatography (IMAC) resins. Protein purity was verified by Coomassie blue staining (Fig. 4B) and Western blot using an anti-poly-His monoclonal antibody (data not shown). Assessment of the ATPase and GTPase activities of wild-type purified CSFV NS4B was performed by evaluating the release of inorganic phosphate using a colorimetric assay as described in Materials and Methods. Consistent with the sequence analysis, purified wild-type NS4B catalyzed the hydrolysis of ATP and GTP (Fig. 4C). Time course analysis of ATP and GTP hydrolysis by wild-type NS4B revealed that the enzymatic activity achieved maximum kinetics after 2 h of incubation with ATP or GTP (Fig. 4C). Data suggest a

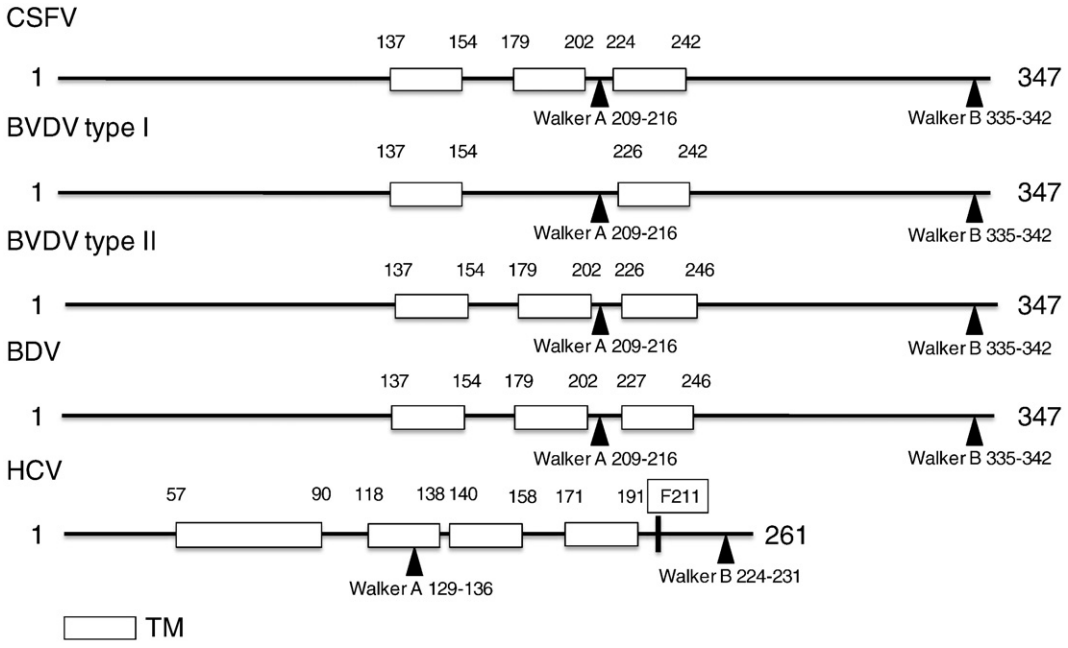

Fig. 1. Schematic representation of NS4B protein from CSFV, BVDV types I and II, BDV, and HCV genotype 1b showing predicted membrane-spanning regions (TM) according to Hofmann and Stoffel, 1993 (TMpred program). Arrowheads indicate locations of Walker A and B motifs. For HCV F211 is the nucleotide binding motif G (Thompson et al., 2009). Numbers indicate amino acid residues.

Accession #	CSFV Strain	Walker A motif	Walker B motif
AY578687	BICv	KSDGLLGTGVS$_{206-216}$	LLGVDSEG$_{335-342}$
X96550	CAP	.....	.....
X87939	Alfort/187	.....	.....
AY578688	CSICv	.....	.....
AY554397	96TD	.....	.....
AY568569	0406/CH/01/TWN	.....	.....
AY775178	Shimen/HVRI	.....	.....
AY805221	C/HVRI	.....	.....
AY259122	Riems	.....	.....
AF326963	Eystrup	.....	.....
AY367767	GXWZ02	.....	.....
AF531433	HCLV	.....	.....
AF407339	39	.....	.....
AF333000	cF114	.....	.....
J04358	HCV	.....	.....
AF091507	HCLV	.....M...	.....
U90951	Alfort A19	.....I...	.....
U45478	Glentorf	.....I...	.....

Fig. 2. Sequence alignment of identified Walker A (amino acid positions 206 to 216) and Walker B (amino acid positions 335 to 342) within CSFV NS4B protein. The Walker A motif consists of a Gly-rich phosphate-binding loop with consensus sequence G/AXXXXGKS/T (where X is any residue). The Walker B motif consists of Asp preceded by a stretch of hydrophobic amino acids hhhhD or hhhhDD/E (where h is a hydrophobic residue) (Walker et al., 1982; Gorbalenya and Koonin, 1989; Mimura et al., 1991; la Cour et al., 1985 and Pai et al., 1989). Notice that the putative Walker A motif indentified within CSFV NS4B lacks the universally conserved Lys residue in the GKS/T signature sequence, where K has been substituted to V.

higher rate of ATP hydrolysis over GTP hydrolysis (30 μM Pi vs. 20 μM Pi, respectively) (Fig. 4C).

Mutations in Walker A and B motifs affect NS4B NTPase activity

To further confirm CSFV NS4B NTPase activity, specific residue substitutions were introduced into the identified Walker A (GLLGTGVS$_{209-216}$) and Walker B (DSEG$_{339-342}$) motifs (Fig. 5), and mutant proteins were tested for their ability to hydrolyze ATP and GTP. Walker A (G209A, L211N, and G214A), Walker B (D339A and G342A), and Walker A/B (G209A, L211N, G214A, D339A, and G342A) mutant proteins demonstrated decreased ATPase and GTPase activities relative to wild-type NS4B (Figs. 6A and B). Overall, mutations in the A motif seem to have a more significant effect on NS4B NTPase activity than mutations introduced in the B motif. To further assess the role of specific residues in the NTPase activity of CSFV NS4B protein, we constructed a Walker A revertant protein harboring an N211L substitution (X_2 position in the canonical A motif) within the backbone of the Walker A mutant (Fig. 5). Interestingly, both ATPase and GTPase activity were partially restored when N211 was reverted back to L in CSFV NS4B protein (Figs. 6C and D). Similar mutations have been shown to affect the NTPase activities of HCV NS4B or Poliovirus 2C proteins (Mirzayan and Wimmer, 1992; Einav et al., 2004). Overall, data confirmed the ATPase and GTPase function of the NS4B protein.

A deviant Walker A motif in CSFV NS4B

Since the identified Walker A motif in CSFV NS4B (GLLGxGVS) deviates from the canonical P-loop sequence (GxxxxGKS/T) by lacking the important phosphate-binding K residue (Figs. 2 and 3B), we substituted conserved K_{206} residues, K206A or K206R, at the N-terminal end of the motif (Fig. 5) and assessed the NTPase activity of the modified proteins. An R residue instead of a K residue is observed at analogous position in NS4B of some BDV isolates (Fig. 3B). Both mutant proteins showed significantly reduced ATPase and GTPase activities relative to wild-type protein (t-test, $P<0.01$). Only K206R mutant protein retained both ATPase and GTPase activities (Figs. 6E and F). These results suggest that in CSFV NS4B the highly conserved K_{206} in the Walker A motif plays a role in NTP hydrolysis. Similar deviations from canonical Walker A motifs have been observed in bacteriophage terminases (Mitchell and Rao, 2004; Tsay et al., 2009) (Fig. 3B).

Effects of NS4B Walker A and Walker B motif disruption on CSFV replication

To assess the role of the Walker A and B motifs in the replication of CSFV a set of mutant viruses was constructed containing amino acid substitutions within the NS4B protein of the CSFV strain Brescia full-length cDNA clone, pBIC. *In vitro* transcribed RNA derived from wild-type and mutant cDNA clones was used to transfect CSFV permissive SK6 cells. Supernatants were harvested and used to infect naive SK6 cell monolayers. CSFV was detected only in cells infected with supernatants derived from wild-type, Walker A revertant, Walker A deviant K206R, and Walker A deviant K206A transfected cells. SK6 cells transfected with RNA transcribed from Walker A mutant, Walker B mutant, and Walker A/B mutant DNA constructs did not yield infectious viruses in three independent attempts (data not shown). Mutations introduced into NS4B were maintained in rescued viruses.

To assess the ability of rescued mutant viruses to replicate *in vitro*, their growth characteristics were evaluated in a multistep growth curve

A

Virus/Protein	Walker A motif								(n)	Walker B motif							
Consensus	*G*	X_1	X_2	X_3	X_4	*G*	*K*	*S/T*	X	H	H	H	H	*D*	X	X	*G*
CSFV/NS4B	*G*	L	L	G	X	*G*	*V*	*S*	(119)	L	L	G	V	*D*	S	E	*G*
BVDV/NS4B	*G*	L	L	G	X	*G*	*I*	*S*	(123)	L	L	G	M	*D*	S	E	*G*
BDV/NS4B	*G*	L	L	G	T	*G*	*V*	*S*	(119)	L	L	G	V	*D*	S	E	*G*
HCV1b/NS4B	*G*	S	I	G	L	*G*	*K*	*V*	(95)	V	P	E	S	*D*	A	A	*A*
HCV3/NS4B	*G*	S	I	G	L	*G*	*R*	*V*	(88)	V	P	E	S	*D*	A	A	*A*
HAV/2C	*G*	K	R	G	G	*G*	*K*	*S*	(41)	V	C	I	I	*D*	D	I	*G*
Polio/2C	*G*	S	P	G	T	*G*	*K*	*S*	(34)	V	V	I	M	*D*	D	L	*N*
FMDV/2C	*G*	K	S	G	Q	*G*	*K*	*S*	(39)	V	V	V	M	*D*	D	L	*G*

B

Virus/Protein	Deviant Walker A motifs										
VHML/gp22	*K*	S	M	R	V	*G*	*Y*	*T*			
Lambda/gpA	*K*	S	A	R	V	*G*	*Y*	*S*			
RM_378/gpPhiN315_66	*K*	P	R	Q	M	*G*	*V*	*T*			
CSFV/NS4B	*K*	S	D	G	L	L	G	X	*G*	*V*	*S*
BVDV/NS4B	*K*	S	D	G	L	L	G	X	*G*	*I*	*S*
BDV/NS4B	*R/K*	S	D	G	L	L	G	T	*G*	*V*	*S*

Fig. 3. (A) Identified Walker A and B motifs within CSFV NS4B are highly conserved among Pestiviruses. Consensus indicates a set of conserved amino acids found in both the GTP-binding members of the G protein superfamily, as well as several viral proteins with nucleotide binding domains. CSFV: classical swine fever virus; BVDV: Bovine Viral Diarrhea Virus; BDV: Border Disease Virus; HCV: Hepatitis C Virus; Polio/2C: Poliovirus 2C protein; HAV2C: Hepatitis A Virus 2C protein; FMDV2C: Foot and Mouth Disease Virus 2C protein. In parenthesis (n) is the number of omitted residues. (B) Alignment of Pestiviruses' Walker A motif with Walker A deviant I motifs (Mitchell and Rao, 2004) found in some bacteriophage proteins where the conserved Lys residue in the GKS/T signature sequence is absent (shaded) and shifted to the beginning of the motif (shaded). VHML: *Vibrio harveyi* bacteriophage; Lambda/gpA: *E. coli* Lambda phage; RM_378gpPhiN315_66: *R* RM_378 *R. marinus* phage.

and compared with parental BICv. All mutant viruses demonstrated growth kinetics similar to BICv although virus titers were at least 0.5 to 1 log lower than the parental virus (Fig. 7). A single substitution (N211L) in a Walker A mutant full-length cDNA clone yielded a Walker A revertant virus, restoring the production of CSFV progeny in SK6 cells. Similarly, substitutions of K206 to A or R, although reducing the NS4B NTPase activity (Figs. 6E and F), do not appear to significantly alter the ability of these viruses to replicate *in vitro*.

Interestingly, these viruses retained a virulent phenotype in infected swine. Animals intramuscularly (IM) inoculated with 10^5 $TCID_{50}$ of any of the mutant viruses (Walker A revertant, Walker A deviant K206R, and Walker A deviant K206A) progressed at the same rate as animals infected with parental BICv in terms of time of death, onset and duration of fever (Table 2), and viremia (Table 3). Viruses isolated from infected pigs retained mutations introduced into NS4B.

Discussion

CSFV non-structural proteins regulate virus replication (Lindenbach et al., 2007; Xu et al., 1997; Moser et al., 1999; Steffens et al., 1999; Xiao et al., 2002, 2006, 2009; Moulin et al., 2007; Sheng et al., 2010). The function of CSFV NS4B protein in viral replication and pathogenesis is poorly understood. Previously, it was found that CSFV NS4B is involved in virulence by modulating the host immune response (Fernandez-Sainz et al., 2010). Here we have shown that CSFV NS4B has ATPase and GTPase activities, and that this activity is mediated by conserved Walker A and Walker B motifs found in the NBM of NTPases (Walker et al., 1982; Gorbalenya and Koonin, 1989). Furthermore, since the identified Walker A motif lacks the universally conserved K residue in the GKS/T signature sequence, we have shown that a highly conserved K_{206} in CSFV NS4B plays *in vitro* a role in ATP and GTP hydrolysis. In that regard, the CSFV NS4B Walker A motif resembles the previously described deviant I Walker A motifs (Mitchell and Rao, 2004).

CSFV NS4B readily hydrolyzes ATP over GTP. Incubation of purified CSFV NS4B with GTP resulted in lower concentrations of Pi relative to the incubation with ATP (Fig. 4C). Similarly, it has been observed that purified His-tagged HCV NS4B hydrolyzes both ATP and GTP, but the protein displayed a higher affinity for ATP and 25-fold faster conversion to ADP than conversion of GTP to GDP (Thompson et al., 2009).

To confirm that ATP and GTP hydrolysis was associated with recombinant CSFV NS4B, a set of mutant proteins was constructed by

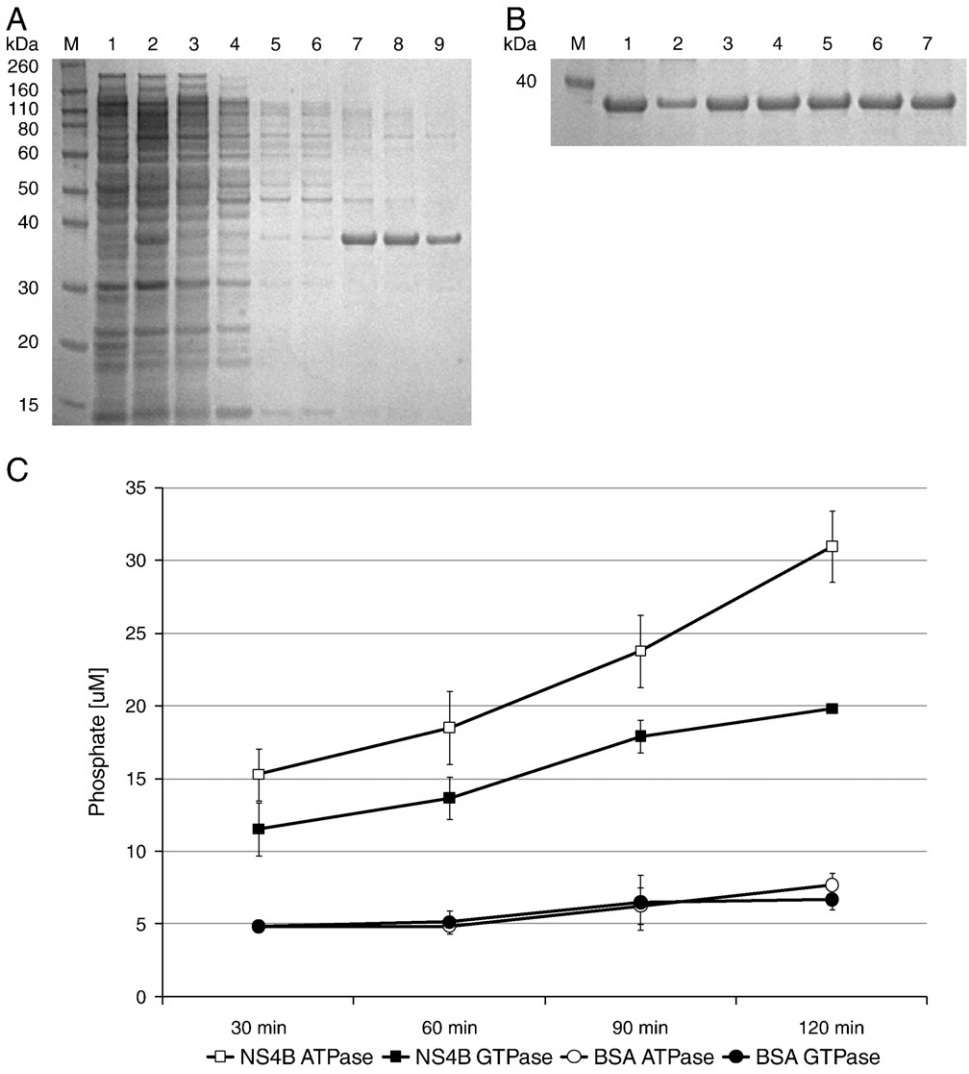

Fig. 4. Purification of *E. coli*-expressed wild-type and mutant CSFV NS4B proteins by immobilized metal ion affinity chromatography (IMAC). (A) Coomassie blue staining of 10% SDS-PAGE showing purification of *E. coli*-expressed wild-type NS4B using HisPur Cobalt Spin Columns. Lanes: M, molecular weight marker (in kDa); 1, cell lysate of BL21(DE3)pLysS transformed with pRSETA vector; 2, cell lysate of BL21(DE3)pLysS transformed with pRSETA encoding wild-type NS4B; 3–6, wash fractions; 7–9, elution fractions containing purified protein. (B) Coomassie blue staining of 10% SDS-PAGE showing purified *E. coli*-expressed wild-type (lane 1) and mutant (lanes 2–7) NS4B. Lanes: M, molecular weight marker (in kDa); 1, wild-type NS4B; 2, Walker A; 3, Walker A revertant; 4, Walker B; 5, Walker A/B; 6, K206R; 7, K206A mutant. (C) ATPase and GTPase activity of wild-type NS4B protein relative to bovine serum albumin (BSA). Proteins were incubated at 30 °C in the presence of Pi-free ATP or GTP and release of Pi (means ± standard deviations from 3 independent experiments) was detected at indicated time points.

introducing amino acid substitutions into the identified A and B motifs. These motifs are involved with binding of phosphate and purine rings of NTP substrates. A significant decrease in NS4B enzymatic activity was observed with Walker A, Walker B, and Walker A/B mutants, demonstrating the NTPase function of the protein (Figs. 6A and B). Further confirmation of the NTPase activity

Virus/Protein	Walker A motif											Walker B motif								
CSFV Brescia	^{206}K	S	D	G	L	L	G	T	G	V	S^{216}	^{335}L	L	G	V	D	S	E	G^{342}	
Walker A mutant	K	S	D	A	L	N	G	T	A	V	S	L	L	G	V	D	S	E	G	
Walker A revertant	K	S	D	A	L	L	G	T	A	V	S	L	L	G	V	D	S	E	G	
Walker B mutant	K	S	D	G	L	L	G	T	G	V	S	L	L	G	V	A	S	E	A	
Walker A/B mutant	K	S	D	A	L	N	G	T	A	V	S	L	L	G	V	A	S	E	A	
Walker A deviant K206R	R	S	D	G	L	L	G	T	G	V	S	L	L	G	V	D	S	E	G	
Walker A deviant K206A	A	S	D	G	L	L	G	T	G	V	S	L	L	G	V	D	S	E	G	

Fig. 5. Set of CSFV NS4B Walker A and Walker B mutant proteins and viruses produced in this study. Underlined are amino acid substitutions.

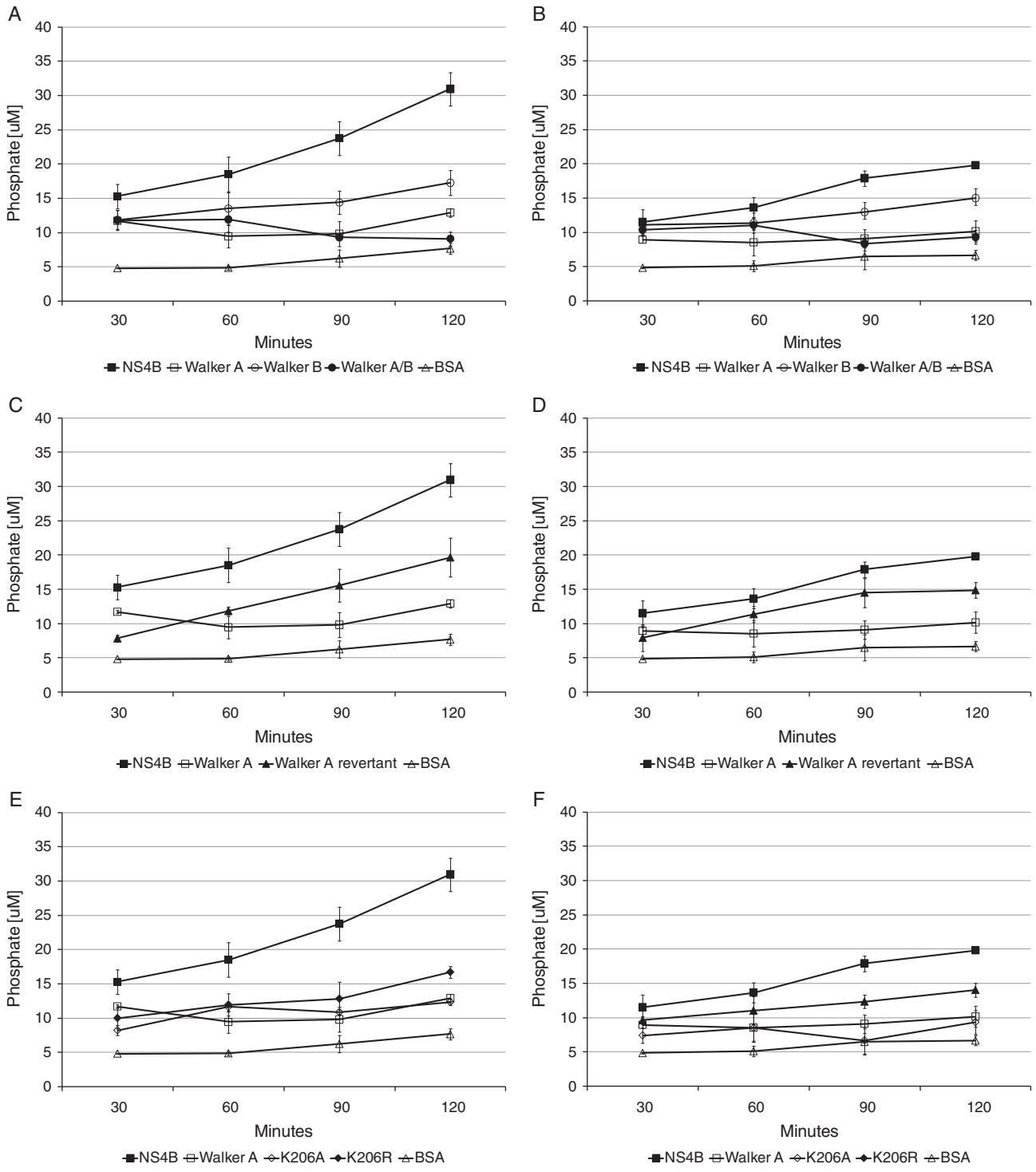

Fig. 6. ATPase (A, C, and E) and GTPase (B, D, and F) activities of wild-type and mutant CSFV NS4B proteins. ATP and GTP hydrolysis of wild-type NS4B relative to Walker A, Walker B, Walker A/B mutants, and BSA (A and B); Walker A revertant NS4B protein (C and D), and Walker A K206A and K206R NS4B mutant proteins (E and F). Assays were performed at 30 °C in the presence of Pi-free ATP or GTP and release of Pi (means ± standard deviations from 3 independent experiments) was detected at 30, 60, 90, and 120 min of incubation.

of NS4B was derived from the Walker A revertant mutant protein. Reversion of N_{211} to wild-type L_{211}, while preserving G209A and G214A mutations, partially restored hydrolysis of ATP and GTP (Figs. 6C and D). The hydrophobic residue at the X_2 position of the A motif appears to have an important effect on NTPase activity, since its mutation in HCV NS4B nearly abolishes GTP hydrolysis (Einav et al., 2004). Furthermore, mutation at an analogous position in poliovirus 2C protein NBM rendered a non-infectious virus (Mirzayan and Wimmer, 1992). The observed changes in CSFV NS4B NTPase activity could be the result of decreased binding and/or decreased hydrolysis of ATP or GTP. In that regard, a double Walker A and B mutant of HCV NS4B was shown to bind both ATP and GTP but with significantly lower affinity while a quintuple mutant was unable to bind either ATP or GTP (Thompson et al., 2009). In the same study, K_{cat} measurements for this double HCV NS4B mutant displayed insignificant changes relative to wild-type, suggesting that these mutations had a strong effect on binding of NTP but little or no effect on the turnover rate.

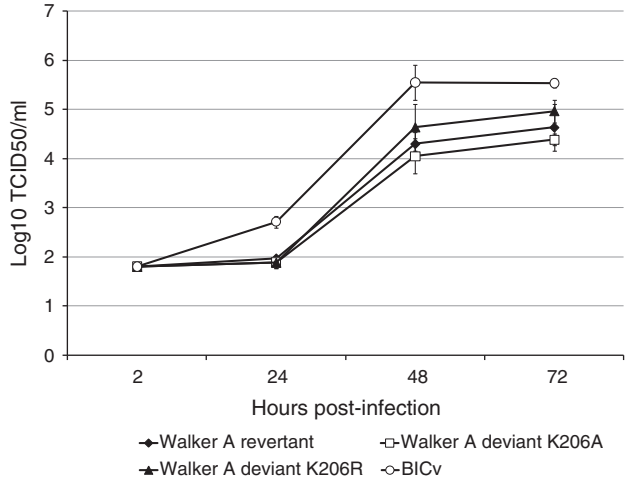

Fig. 7. *In vitro* growth characteristics of NS4B mutants and parental BICv. SK6 cell cultures were infected (MOI = 0.01) with each of the mutants or BICv and virus yield titrated at times post infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: $\geq \log_{10}$ 1.8 TCID50/ml.

The Walker A motif is one of the most common and highly conserved motifs found in genomes (Koonin, 1993a). The identified A motif in CSFV NS4B as well as in other pestiviruses lacks the universally conserved K residue in the GKS/T signature. In the signature sequence, the K residue interacts with the negatively charged β- and γ-phosphates of NTP (la Cour et al., 1985; Pai et al., 1989, 1990). Large terminase proteins (subunits of DNA packing machines in large DNA viruses), including the well-characterized λ phage gpA and SPP1 G2P, lack a canonical Walker A motif. However, a conserved K is consistently located at the N-terminal end of the putative Walker A motifs of all these phage terminases (Mitchell and Rao, 2004). These non-canonical Walker A motifs are known as deviant I Walker A motifs. Here we confirmed that K_{206} is important for NS4B hydrolysis of NTPs, suggesting that pestiviruses may harbor a deviant I Walker A motif in NS4B. However, viruses harboring substitutions of the conserved K_{206} were able to replicate in swine macrophages and retained their virulent phenotype in infected swine, suggesting that in the context of the virus infection this residue is not critical for replication. Nonetheless, this finding may have important implications for developing novel antiviral compounds against CSFV.

We have observed that disruption of predicted Walker A and B motifs in CSFV NS4B significantly affected virus replication. However, replication of the mutant Walker A virus was rescued by introducing an N211L substitution. This virus retained the capability to cause disease in swine, suggesting that L_{211} is critical for the NTPase activity of the virus, and that the NTPase activity of NS4B plays a key role in virus viability and virulence. Binding and hydrolysis of nucleotides by viral proteins mediates a variety of events involved with virus replication including signaling, membrane trafficking, and membrane fusion. For instance, Poliovirus nonstructural 2C protein is an ATPase found in abundance in the membranous replication complex where viral RNA synthesis occurs (Cho et al., 1994; Teterina et al., 1992). Analogous to picornaviral 2C proteins, HCV NS4B possesses NTPase activity and is involved in the reorganization of intracellular membranes and virus replication (Pfister and Wimmer, 1999; Samuilova et al., 2006; Thompson et al., 2009). Furthermore, mutations in the NBM of poliovirus 2C and HCV NS4B impaired viral replication (Teterina et al., 1992; Mirzayan and Wimmer, 1992; Einav et al., 2004).

In summary, we predicted, detected, and mapped residues that confer NTPase activity to CSFV NS4B protein. Furthermore, we showed that residues outside the canonical Walker A motif are important for the *in vitro* enzymatic activity of NS4B, as in described deviant type I Walker A motifs. The NTPase activity of this protein is required for CSFV replication. The defined motifs may represent specific targets for anti-CSFV compounds.

Table 1
Sequences of oligonucleotide primers used for generation of wild-type (wt) and mutant CSFV NS4B protein and mutant viruses.

Primer	Sequence (5′→3′)
wt NS4B for	GGATCCGCTCAGGGGGATGTGCAGAGATGT
wt NS4B rev	CCATGGTTATAGCTGGCGGATCTTTCCTTC
Walker A for	GAAAAAGCGATGCTTTGAACGGCACAGCGGTTAGTGCGGCTATGG
Walker A rev	CCATAGCCGCACTAACCGCTGTGCCGTTCAAAGCATCGCTTTTTC
Walker A revertant for	GAAAAAGCGATGCTTTGCTAGGCACAGCGGTTAGTGCGGCTATGG
Walker A revertant rev	CCATAGCCGCACTAACCGCTGTGCCTAGCAAAGCATCGCTTTTTC
Walker B for	GAACTACTGGGAGTAGCTAGCGAAGCAAAGATCCGCCAGCTA
Walker B rev	TAGCTGGCGGATCTTTGCTTCGCTAGCTACTCCCAGTAGTTC
K206R for	TCAATCAGGCGCGGAAGAAGCGATGCTTTG
K206R rev	CAAAGCATCGCTTCTTCCGCGCCTGATTGA
K206A for	TCAATCAGGCGCGGAGCAAGCGATGCTTTG
K206A rev	CAAAGCATCGCTTGCTCCGCGCCTGATTGA

Codon changes are underlined; for, forward primer; rev, reverse primer.

Materials and methods

Cloning, expression, purification, and detection of wild-type and mutant NS4B proteins

The gene encoding for CSFV strain Brescia NS4B protein (1041 bp) was amplified by polymerase chain reaction (PCR) using specific primers (Table 1). The resulting PCR fragment was cloned into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA). The plasmid was subsequently digested with *BamH*I and *Nco*I and the expected 1041 bp NS4B fragment was directionally cloned into the pRSETA expression plasmid (Invitrogen). Site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA) was utilized to generate mutant NS4B genes using primers described in Table 1. Complete sequences of all DNA constructs were verified by automated sequencing.

One Shot BL21 (DE3) pLysS *E. coli* (Invitrogen) cells were transformed with 10 ng of pRSETA plasmid encoding wild-type or mutant NS4B, cultured in MagicMedia (Invitrogen) for 21 h at 37 °C with shaking, and harvested by centrifugation at 3200 ×g for 15 min. Cell pellets were then treated with CelLytic buffer (Sigma Aldrich, St. Louis, MO) according to the manufacturer's large scale extraction protocol. For His-tagged protein purification, cell lysates were applied to HisPur Cobalt Spin Columns (Thermo Fisher Scientific, Rockford, IL) and the collected fractions were analyzed on a 10% NuPage Novex Bis–Tris gel (Invitrogen) using a discontinuous SDS-PAGE system. Western blots were performed using polyvinylidene fluoride (PVDF) membranes (Invitrogen) and a His-Tag antibody (Novagen, EMD Biosciences, Madison, WI). Reactions were developed using alkaline phosphatase labeled goat anti-mouse IgG antibody with the WesternBreeze Chemiluminescent Detection Kit (Invitrogen). The purified proteins were desalted twice using 7K MWCO Zebra Spin Desalting Columns (Thermo Fisher Scientific). Protein concentration was determined by BCA Protein Assay (Thermo Fisher Scientific) against known standards at OD of 595 nm using a NanoDrop instrument (Thermo Scientific NanoDrop Products, Wilmington, DE).

NTPase assays

The ATPase and GTPase activities of recombinant wild-type and mutant CSFV NS4B proteins were determined using the Bioassay Systems colorimetric ATPase/GTPase assay system according to the

Table 2
Swine survival and fever response following infection with CSFV NS4B mutants and parental BICv.

Virus	No. of survivors/ total no.	Mean time to death (days ± SD)	Fever		
			No. of days to onset (days ± SD)	Duration no. of days (days ± SD)	Max daily temperature (°F ± SD)
Walker A revertant	0/3	7.7 (0.6)	3.7 (0.6)	4.7 (0.6)	105.2 (0.8)
Walker A deviant K206A	0/3	9 (1)	3.5 (0.6)	5.7 (1.2)	106.5 (1.2)
Walker A deviant K206R	0/3	8.7 (0.6)	3 (0)	4.7 (0.6)	105.9 (0.8)
BICv	0/3	9 (1)	3 (0)	6 (1)	107.0 (0.8)

manufacturer's recommendations (QuantiChrom ATPase/GTPase Assay Kit, BioAssay Systems, Hayward, CA). Briefly, NTPase assays were performed with 1.5 μg of protein in 20 μl of Assay Buffer (40 mM Tris, 80 mM NaCl, 8 mM $MgAc_2$, and 1 mM EDTA, pH 7.5). Forty mM of ATP or GTP was added to each well and the reaction was incubated for 30, 60, 90, or 120 min at 30 °C. The assay contained purified inorganic phosphate (Pi)-free NTP substrate to ensure lowest possible background signals. Following incubation with ATP or GTP, 200 μl of BioAssay Systems Reagent were added and the reaction mixtures were developed for 30 min. Samples were read at 635 nm on a Synergy HT Microplate reader (BioTek, Winooski, VT). Phosphate standards provided with the kit were used to generate a standard curve and to calculate the enzymatic activity of wild-type and mutant NS4B proteins. Purified Pi-free Bovine Serum Albumin (Sigma, St. Louis, MO) was used in the assays as a negative control. The results were determined by calculating the amount of Pi produced by the enzyme after incubation with the substrate for the indicated period of time.

Construction of CSFV mutants

A full-length cDNA infectious clone (IC) of virulent CSFV strain Brescia (pBIC) (Risatti et al., 2005) was used as a template in which putative NBM sites in the NS4B gene were mutated. Mutations were introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) performed per manufacturer's instructions and using the primers described in Table 1. Full-length genomic clones were linearized with *Srf*I and *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX). RNA was precipitated with LiCl and transfected into SK6 cells (Terpstra et al., 1990) by electroporation at 500 V, 720 Ω, and 100 W with a BTX 630 electroporator (BTX, San Diego, CA). Cells were cultured in Dulbecco's minimal essential media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO), seeded in 6-well plates and incubated for 4 days at 37 °C and 5% CO_2. Effectiveness of the transfection was assessed by immunoperoxidase staining using CSFV E2 monoclonal antibody WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Transfected cultures were harvested at day 4 post-transfection and the presence of infectious virus in the supernatant was detected by seeding the material on fresh SK6 cell cultures. After 4 days, cells were fixed and the presence of E2 expression was assessed by immunoperoxidase staining as described above. Virus growth characteristics were evaluated in multistep growth curves. SK6 cells were infected at a MOI = 0.01 $TCID_{50}$ and virus adsorbed for 1 h (time zero). Samples were then collected at times post-infection through 72 h, and virus yields titrated on SK6 cells. Virus titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was ≥ 1.8 $TCID_{50}$/ml.

Table 3
Viremia in pigs inoculated with parental BICv and NS4B mutant viruses.

	Days post infection			
	4	6	8	9
Walker A rev virus				
Pig 1	3.97	5.63	7.1	D
Pig 2	3.80	5.80	6.80	D
K206R virus				
Pig 1	3.80	5.97	6.97	D
Pig 2	3.63	5.63	6.63	D
K206A virus				
Pig 1	3.63	5.80	6.97	D
Pig 2	3.80	5.97	7.10	D
BICv				
Pig 1	3.80	5.80	7.10	D
Pig 2	4.00	5.97	6.97	D

Values expressed as $\log_{10}$ TCID50/ml. D: deceased.

DNA sequencing and analysis

All DNA clones were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Mutant viruses isolated from infected animals were sequenced in the region where changes were introduced.

Animal experiments

Mutant viruses recovered from transfected SK6 cells were screened for their virulence phenotype in swine relative to the virulent Brescia strain. Swine used in these studies were 10 to 12 week old, forty-pound commercial breed pigs inoculated intramuscularly (IM) with 10^5 $TCID_{50}$ of mutant viruses or wild-type parental virus (BICv). Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the 21-day experiment.

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References

Cho, M.W., Teterina, N., Egger, D., Bienz, K., Ehrenfeld, E., 1994. Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. Virology 202 (1), 129–145.

Dever, T.E., Glynias, M.J., Merrick, W.C., 1987. GTP-binding domain: three consensus sequence elements with distinct spacing. Proc. Natl Acad. Sci. USA 84 (7), 1814–1818.
Edwards, S., Moennig, V., Wensvoort, G., 1991. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. Vet. Microbiol. 29 (2), 101–108.
Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H.E., Moradpour, D., Bienz, K., 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. 76 (12), 5974–5984.
Einav, S., Elazar, M., Danieli, T., Glenn, J.S., 2004. A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. J. Virol. 78 (20), 11288–11295.
Einav, S., Sklan, E.H., Moon, H.M., Gehrig, E., Liu, P., Hao, Y., Lowe, A.W., Glenn, J.S., 2008. The nucleotide binding motif of hepatitis C virus NS4B can mediate cellular transformation and tumor formation without Ha-ras co-transfection. Hepatology 47 (3), 827–835.
Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., Ed. 2005. Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. Edited by M. A. M. C.M. Fauquet, J. Maniloff, U. Desselberger, and L.A. Ball (Elsevier Academic Press): ICTV.
Fernandez-Sainz, I., Gladue, D.P., Holinka, L.G., O'Donnell, V., Gudmundsdottir, I., Prarat, M.V., Patch, J.R., Golde, W.T., Lu, Z., Zhu, J., Carrillo, C., Risatti, G.R., Borca, M.V., 2010. Mutations in classical swine fever virus NS4B affect virulence in swine. J. Virol. 84 (3), 1536–1549.
Gong, Y., Trowbridge, R., Macnaughton, T.B., Westaway, E.G., Shannon, A.D., Gowans, E.J., 1996. Characterization of RNA synthesis during a one-step growth curve and of the replication mechanism of bovine viral diarrhoea virus. J. Gen. Virol. 77 (Pt 11), 2729–2736.
Gorbalenya, A.E., Koonin, E.V., 1989. Viral proteins containing the purine NTP-binding sequence pattern. Nucleic Acids Res. 17 (21), 8413–8440.
Hofmann, K., Stoffel, W., 1993. TMbase — a database of membrane spanning proteins segments. Biol. Chem. Hoppe Seyler 374, 166.
Hugle, T., Fehrmann, F., Bieck, E., Kohara, M., Krausslich, H.G., Rice, C.M., Blum, H.E., Moradpour, D., 2001. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. Virology 284 (1), 70–81.
Kim, M., Mackenzie, J.M., Westaway, E.G., 2004. Comparisons of physical separation methods of Kunjin virus-induced membranes. J. Virol. Meth. 120 (2), 179–187.
Koonin, E.V., 1993a. A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. Nucleic Acids Res. 21 (11), 2541–2547.
Koonin, E.V., 1993b. A superfamily of ATPases with diverse functions containing either classical or deviant ATP-binding motif. J. Mol. Biol. 229 (4), 1165–1174.
la Cour, T.F., Nyborg, J., Thirup, S., Clark, B.F., 1985. Structural details of the binding of guanosine diphosphate to elongation factor Tu from *E. coli* as studied by X-ray crystallography. EMBO J. 4 (9), 2385–2388.
Lindenbach, B.D., Thiel, H.-J., Rice, C.M., 2007. Flaviviridae: the viruses and their replication, In: Knipe, David M., Howley, Peter M., Griffin, Diane E., Lamb, Robert A., Martin, Malcolm A., Bernard, Roizman, Straus, Stephen E. (Eds.), Fundamental Virology, Fifth ed. Lippincott Williams & Wilkins, pp. 1001–1152.
Lindstrom, H., Lundin, M., Haggstrom, S., Persson, M.A., 2006. Mutations of the hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons. Virus Res. 121 (2), 169–178.
Miller, S., Sparacio, S., Bartenschlager, R., 2006. Subcellular localization and membrane topology of the Dengue virus type 2 Non-structural protein 4B. J. Biol. Chem. 281 (13), 8854–8863.
Mimura, C.S., Holbrook, S.R., Ames, G.F., 1991. Structural model of the nucleotide-binding conserved component of periplasmic permeases. Proc. Natl Acad. Sci. USA 88 (1), 84–88.
Mirzayan, C., Wimmer, E., 1992. Genetic analysis of an NTP-binding motif in poliovirus polypeptide 2C. Virology 189 (2), 547–555.
Mitchell, M.S., Rao, V.B., 2004. Novel and deviant Walker A ATP-binding motifs in bacteriophage large terminase-DNA packaging proteins. Virology 321 (2), 217–221.
Moser, C., Stettler, P., Tratschin, J.D., Hofmann, M.A., 1999. Cytopathogenic and noncytopathogenic RNA replicons of classical swine fever virus. J. Virol. 73 (9), 7787–7794.
Moulin, H.R., Seuberlich, T., Bauhofer, O., Bennett, L.C., Tratschin, J.D., Hofmann, M.A., Ruggli, N., 2007. Nonstructural proteins NS2-3 and NS4A of classical swine fever virus: essential features for infectious particle formation. Virology 365 (2), 376–389.
Pai, E.F., Kabsch, W., Krengel, U., Holmes, K.C., John, J., Wittinghofer, A., 1989. Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation. Nature 341 (6239), 209–214.
Pai, E.F., Krengel, U., Petsko, G.A., Goody, R.S., Kabsch, W., Wittinghofer, A., 1990. Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. EMBO J. 9 (8), 2351–2359.
Pfister, T., Wimmer, E., 1999. Characterization of the nucleoside triphosphatase activity of poliovirus protein 2C reveals a mechanism by which guanidine inhibits poliovirus replication. J. Biol. Chem. 274 (11), 6992–7001.
Reed, L.J., Muench, H.A., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.
Risatti, G.R., Borca, M.V., Kutish, G.F., Lu, Z., Holinka, L.G., French, R.A., Tulman, E.R., Rock, D.L., 2005. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. J. Virol. 79 (6), 3787–3796.
Rumenapf, T., Unger, G., Strauss, J.H., Thiel, H.J., 1993. Processing of the envelope glycoproteins of pestiviruses. J. Virol. 67 (6), 3288–3294.
Samuilova, O., Krogerus, C., Fabrichniy, I., Hyypia, T., 2006. ATP hydrolysis and AMP kinase activities of nonstructural protein 2C of human parechovirus 1. J. Virol. 80 (2), 1053–1058.
Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl Acad. Sci. USA 74 (12), 5463–5467.
Sheng, C., Zhu, Z., Yu, J., Wan, L., Wang, Y., Chen, J., Gu, F., Xiao, M, 2010. Characterization of NS3, NS5A and NS5B of classical swine fever virus through mutation and complementation analysis. Vet. Microbiol. 140(1–2), 72–80.
Steffens, S., Thiel, H.J., Behrens, S.E., 1999. The RNA-dependent RNA polymerases of different members of the family Flaviviridae exhibit similar properties in vitro. J. Gen. Virol. 80 (Pt 10), 2583–2590.
Suzich, J.A., Tamura, J.K., Palmer-Hill, F., Warrener, P., Grakoui, A., Rice, C.M., Feinstone, S.M., Collett, M.S., 1993. Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. J. Virol. 67 (10), 6152–6158.
Tamura, J.K., Warrener, P., Collett, M.S., 1993. RNA-stimulated NTPase activity associated with the p80 protein of the pestivirus bovine viral diarrhea virus. Virology 193 (1), 1–10.
Tautz, N., Elbers, K., Stoll, D., Meyers, G., Thiel, H.J., 1997. Serine protease of pestiviruses: determination of cleavage sites. J. Virol. 71 (7), 5415–5422.
Terpstra, C., Woortmeyer, R., Barteling, S.J., 1990. Development and properties of a cell culture produced vaccine for hog cholera based on the Chinese strain. Dtsch Tierärztl. Wochenschr. 97 (2), 77–79.
Teterina, N.L., Kean, K.M., Gorbalenya, A.E., Agol, V.I., Girard, M., 1992. Analysis of the functional significance of amino acid residues in the putative NTP-binding pattern of the poliovirus 2C protein. J. Gen. Virol. 73 (Pt 8), 1977–1986.
Thiel, H.J., Plagemann, P.G.W., Moennig, V., 1996. Pestiviruses, In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fundamental Virology, 3rd ed. Lippincott-Raven, Philadelphia, pp. 1059–1073.
Thompson, A.A., Zou, A., Yan, J., Duggal, R., Hao, W., Molina, D., Cronin, C.N., Wells, P.A., 2009. Biochemical characterization of recombinant hepatitis C virus nonstructural protein 4B: evidence for ATP/GTP hydrolysis and adenylate kinase activity. Biochemistry 48 (5), 906–916.
Tsay, J.M., Sippy, J., Feiss, M., Smith, D.E., 2009. The Q motif of a viral packaging motor governs its force generation and communicates ATP recognition to DNA interaction. Proc. Natl Acad. Sci. USA 106 (34), 14355–14360.
Walker, J.E., Saraste, M., Runswick, M.J., Gay, N.J., 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1 (8), 945–951.
Wang, P., Wang, Y., Zhao, Y., Zhu, Z., Yu, J., Wan, L., Chen, J., Xiao, M., 2010. Classical swine fever virus NS3 enhances RNA-dependent RNA polymerase activity by binding to NS5B. Virus Res. 148 (1–2), 17–23.
Weiskircher, E., Aligo, J., Ning, G., Konan, K.V., 2009. Bovine viral diarrhea virus NS4B protein is an integral membrane protein associated with Golgi markers and rearranged host membranes. Virol. J. 6, 185.
Wen, G., Chen, C., Luo, X., Wang, Y., Zhang, C., Pan, Z., 2007. Identification and characterization of the NTPase activity of classical swine fever virus (CSFV) nonstructural protein 3 (NS3) expressed in bacteria. Arch. Virol. 152 (8), 1565–1573.
Wen, G., Xue, J., Shen, Y., Zhang, C., Pan, Z., 2009. Characterization of classical swine fever virus (CSFV) nonstructural protein 3 (NS3) helicase activity and its modulation by CSFV RNA-dependent RNA polymerase. Virus Res. 141 (1), 63–70.
Xiao, M., Zhang, C.Y., Pan, Z.S., Wu, H.X., Guo, J.Q., 2002. Classical swine fever virus NS5B-GFP fusion protein possesses an RNA-dependent RNA polymerase activity. Arch. Virol. 147 (9), 1779–1787.
Xiao, M., Li, H., Wang, Y., Wang, X., Wang, W., Peng, J., Chen, J., Li, B., 2006. Characterization of the N-terminal domain of classical swine fever virus RNA-dependent RNA polymerase. J. Gen. Virol. 87 (Pt 2), 347–356.
Xiao, M., Wang, Y., Zhu, Z., Yu, J., Wan, L., Chen, J., 2009. Influence of NS5A protein of classical swine fever virus (CSFV) on CSFV internal ribosome entry site-dependent translation. J. Gen. Virol. 90 (Pt 12), 2923–2928.
Xu, J., Mendez, E., Caron, P.R., Lin, C., Murcko, M.A., Collett, M.S., Rice, C.M., 1997. Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. J. Virol. 71 (7), 5312–5322.